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Authors	Cormican, David;Kennedy, Ciaran;Murphy, Sandra;Werner, Reiltin;Power, Derek G.;Heffron, Cynthia C. B. B.
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**University College Cork, Ireland**  
Coláiste na hOllscoile Corcaigh

**Title:** High Concordance of BRAF Mutational Status in Matched Primary and Metastatic Melanoma

**Short Running Title:** High Concordance of BRAF in Melanoma

**List of Authors:**

David Cormican<sup>1,2</sup> (davidlcormican@gmail.com)

Ciaran Kennedy<sup>1</sup> (ciaran.kennedy@hse.ie)

Sandra Murphy<sup>1</sup> (sandra.murphy4@hse.ie)

Reiltin Werner<sup>1</sup> (reiltin.werner@hse.ie)

Derek G. Power<sup>3,4</sup> (derek.power1@hse.ie)

Cynthia CBB Heffron<sup>1,5</sup> (cynthia.heffron@hse.ie)

**Corresponding Author:** Cynthia CBB Heffron

**Postal Address (Corresponding Author):** Dr. Cynthia Heffron, Department of Histopathology, Cork University Hospital, Wilton, Cork

**Phone Number (Corresponding Author):** +353 (0)21 4920485

**E-mail (Corresponding Author):** [cynthia.heffron@hse.ie](mailto:cynthia.heffron@hse.ie)

**Author Affiliations**

1. Department of Pathology, Cork University Hospital, Wilton, Cork, Ireland
2. Department of Histopathology, School of Medicine, Trinity College, Dublin, Ireland
3. Department of Medical Oncology, Cork University Hospital, Wilton, Cork, Ireland
4. Department of Medicine, School of Medicine, University College Cork, Cork, Ireland
5. Department of Pathology, School of Medicine, University College Cork, Cork, Ireland

**Keywords:** Melanoma, BRAF, Immunohistochemistry, Metastasis

**Abstract**

Background: Techniques for the accurate identification of activating mutations of BRAF in metastatic melanoma are of great clinical importance, due to the availability of targeted therapies for these tumours. There is uncertainty regarding the frequency with which BRAF status differs between primary and metastatic sites.

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Methods: Between 2011 and 2016, 219 melanoma cases underwent BRAF testing in our institution. In 53 of these, paired primary and metastatic specimens were available for PCR and immunohistochemical evaluation.

Results: 52 out of 53 cases (98%) showed concordant BRAF status between primary and metastatic site by IHC. In one case, a metastasis and its matched primary were positive by IHC but the metastasis was negative on PCR. On further investigation, PCR was positive in the primary and repeat PCR in the metastasis was positive, following macrodissection.

Conclusions: Our results suggest that discordance of BRAF mutational status between primaries and metastases is a rare occurrence. In one case, immunohistochemistry provided strong evidence that initial PCR testing had provided a false negative result due to low tumour volume. Thus, in cases where tissue is difficult to obtain from a metastasis or unavailable, the primary tumour can be used with confidence.

Key Words: Melanoma, BRAF, Immunohistochemistry, Metastasis

## Introduction

Cutaneous malignant melanoma is an invasive, neoplastic proliferation of the melanocytes of the skin. According to World Health Organisation data, there are approximately 232,000 cases of melanoma diagnosed per year; with the highest incidence rates seen in Australia and New Zealand, North America, and Northern and Western Europe. (1)

The landscape of somatic genetic mutations in melanoma is complex and variable between patients. (2) Gain-of-function mutations of BRAF (a signal transducer for a number of growth factor receptors) were shown to be of significance in a significant proportion of human cancer by Davies et al in 2002. (3, 4) Subsequent studies suggest that BRAF mutations are present in 40 – 60% of melanoma. (5-8) The most common mutant form of BRAF in human cancer is BRAF V600E, which accounts for over 90% of BRAF mutations in melanoma. (9) A small number of melanomas have an alternative substitution at position BRAF codon 600 (for example BRAF V600K). (9)

The emergence of effective targeted therapy against constitutively active BRAF has made the ability to accurately determine BRAF mutational status in melanoma critical to directing therapy. Vemurafenib and dabrafenib are small molecule inhibitors of mutated BRAF, which have been shown to extend life in individuals with stage IV metastatic melanoma. (10) However, these drugs are ineffective in cases lacking BRAF mutation. Survival in BRAF mutated disease is improved by addition of trametinib to inhibit the activity of MEK, a downstream protein in the BRAF signalling cascade. (11)

The current gold standard for testing BRAF mutational status in melanoma is real time polymerase chain reaction (RT-PCR), which indicates whether there is a mutation present in BRAF gene at the DNA level. (12) More recently, the development of a monoclonal antibody specific for BRAF V600E has made it feasible to test for mutant BRAF protein by immunohistochemistry (IHC). Multiple studies have demonstrated high concordance between results of testing by RT-PCR and IHC. (13, 14) RT-PCR is more widely validated, and may identify rare mutations (such as V600K) which are not detectable on IHC but predict response to BRAF inhibition. However, IHC may provide information on the strength and pattern of mutant protein expression which commercially available PCR assays cannot provide. Furthermore, IHC may be more accurate for small melanoma deposits where false negatives may occur on PCR if tumour cells relatively small percentage of the sampled tissue. (15) Therefore, IHC and RT-PCR may have complementary roles in the evaluation of BRAF mutational status in melanoma in the research setting.

An unresolved issue in BRAF testing is the frequency with which BRAF status of a melanoma primary differs from that of metastases to which it gives rise. A recent meta-analysis of studies examining non-concordance of BRAF status between sites by various methods concluded that 'a clinically meaningful discrepancy rate of approximately 13.4% existed'. (16) Current European Society of Medical Oncology guidelines recommend that tissue from a metastatic lesion be used for BRAF testing in preference to primary tumour tissue when both are available. (17) Best practice where only primary tissue is available remains undefined, but the authors of the meta-analysis advocate a second procedure to biopsy a metastatic site under these circumstances. (16)

It is noteworthy that in previous work on BRAF status discordance between primary and metastatic melanoma, pooled rate of discordance was lower in studies using IHC to detect mutant protein, compared to those using DNA-based approaches. (16) Across seven studies using the VE1 anti-V600E BRAF antibody, the mean discordance was 5.6%. (18-24) By contrast, the mean discordance was 14% in DNA-based studies. (16) The reasons for this difference remain obscure, and could include technical issues around use of the relatively recently developed VE1 antibody. Our centre has significant previous experience in the application of VE1. (13) In view of this, we undertook the current study to investigate BRAF concordance by IHC between matched melanoma metastases and primary tumours to establish the rate of discordance in an Irish population.

## Methods

### Patient Selection

We sought to include patients treated for melanoma at our institution (Cork University Hospital) between 2012 and 2016 who fulfilled the following criteria: (1) Biopsy-proven melanoma primary; (2) Biopsy-proven melanoma metastasis of at least one site (regional lymph node, cutaneous metastasis, or distant visceral

metastasis); (3) PCR sequencing to establish BRAF mutational status carried out on at least one site; and (4) Sufficient remaining tissue available from both primary and metastatic site to allow immunohistochemistry to be performed.

Initial search of electronic patient records found 218 melanoma patients had undergone PCR testing to establish BRAF status. Of these, 72 had matched biopsy samples from primary and metastatic site. In nineteen cases, there was insufficient tissue from one or both sites to allow further testing, leaving a final cohort of 53 patients.

### **PCR Sequencing**

All patients had undergone BRAF V600E PCR sequencing by cobas © 4800 BRAF V600 mutation test prior to inclusion in our study as clinically required.

### **BRAF V600E Immunohistochemistry**

Immunohistochemistry for the BRAF V600E mutation was performed using the Roche Ventana anti-BRAF V600E VE1 clone antibody on the Ventana Benchmark Ultra platform slide staining system. This antibody was validated and optimised for use by Roche Diagnostics offsite prior to the commencement of the study. The process involved cell conditioning for 64 minutes (Tris based buffer), pre oxidation inhibition and primary antibody incubation for 16 minutes at 36.C. Ventana Optiview DAB IHC detection kit was used to detect BRAF V600E protein expression. The slides were counterstained with Ventana Haematoxylin and Bluing agent for 4 minutes.

### **Scoring of BRAF Immunohistochemistry**

Immunohistochemistry was reviewed by a consultant histopathologist who was blinded to the PCR result and immunohistochemistry result at the matched second site. Cases were regarded as positive if there was any evidence of cytoplasmic BRAF staining (1+, 2+, or 3+; see Figure 1). This interpretation was used by our group previously in the validation of BRAF IHC. (13)

### **Ethical Approval**

Ethical approval for this study was granted by Cork University Hospital's Clinical Research Ethics Committee on 18<sup>th</sup> November 2014. The application reference number was ECM 4(nnn) 19/11/14.

## **Results**

### **Clinicopathological Characteristics of the Cohort**

The cases included in the cohort included primaries and metastases from a wide range of sites and a number of histological subtypes were represented (see Table 1). Median age at first biopsy was 69 years and the median time from biopsy of primary to biopsy of metastasis was 9 months (range 0 to 83 months).

### **Primary-Metastatic Concordance by Immunohistochemistry**

In all cases, the primary and metastatic tumours were concordant in terms of BRAF status as assessed by immunohistochemistry. 10 of 53 cases (19%) were positive for BRAF V600E mutant protein at the primary and metastatic site, while 43 were negative at both sites.

### **Concordance of Immunohistochemistry and PCR Results**

BRAF V600E mutation was detected by PCR in 9 of 53 cases (19%), with one further case positive for BRAF V600K. The V600K mutated case was negative for mutated BRAF expression by IHC, as was expected based on previous studies using the VE1 antibody. One case was positive for expression of V600E by IHC while lacking evidence of a genetic mutation by PCR and we undertook further investigation to explain this unexpected finding.

### **Further Characterisation of PCR/Immunohistochemistry Discordant Cases**

One case in which mutation was detected by PCR but not by immunohistochemistry exhibited the unusual BRAF V600K variant according to PCR results. Thus the negative IHC result could be explained in terms of the limits of current IHC techniques, which were previously outlined.

One case which was positive by IHC at both sites but in which PCR had failed to detect mutation in a metastatic deposit required further investigation. Firstly PCR of the primary lesion was undertaken and was positive for mutation. Subsequently, review of haematoxylin and eosin stained sections of the metastatic deposit demonstrated a low volume of tumour; the metastasis tested had a maximum dimension of 1.5mm and was present in a subcapsular location within a much larger lymph node. We speculated that negativity of the initial PCR carried out on the metastasis may have been due to technical issues related to the low volume of tumour compared to overall tissue volume. With this in mind, we carried out macrodissection on the metastatic sample to isolate the metastatic lesion from the background nodal tissue and then repeated PCR. Repeat PCR for the macrodissected metastatic tumour was positive for BRAF V600E mutation, strongly suggesting that the initial result was a false negative caused by technical issues with the PCR assay and related to the low volume of tumour at the site tested.

### **Discussion**

The ability of clinical diagnostic laboratories to accurately and rapidly determine the presence or absence of BRAF mutation in metastatic melanoma is vital to ensure patients receive correct and timely therapy. Any issue impacting on laboratory testing of mutational status is of urgent research interest. We undertook this study to address one such issue; specifically the question of how frequently BRAF mutational status is discordant between a primary melanoma and the distant metastases to which it gives rise.

Previous work in this area has produced widely different results regarding the frequency of such discordance. Menzies *et al* and Manfredi *et al* reported that BRAF status of melanoma metastases as assessed by IHC were identical to that of the matched primary in all cases in series of 64 and 54 patients, respectively. (20, 21) A very recent study by Nielsen *et al* included 82 patients with primary and metastatic melanoma with all but one case concordant. (22) The largest study to use IHC to compare BRAF status between primary melanomas and their metastases was Boursault *et al*, which included 88 patients and reported discordance in 4.5%. (18) While rates of discordance on IHC as high as 27.5% have been reported (23), no study including more than 50 patients has shown discordance in more than 5% of cases. (16, 18-21) The characteristics of previous studies addressing this issue by means of IHC are summarised in Table 2.

In contrast, rates of discordance of over 40% have been reported in some small studies ( $n < 20$ ) using DNA-based methods such as PCR. (26, 27) The largest PCR study included 236 patients and reported discordance in 11.8% of cases. (28) Other studies using DNA-based methods have reported rates between 4% and 47%. (18, 20, 29-36) In their recent meta-analysis, Valachis and Ullenhag found a significantly lower pooled rate of discordance in IHC-based studies (5%) compared to those using DNA-based methods (14.3%). (16) Nonetheless, the authors of that analysis conclude that the average rate of discordance across all studies is sufficient to indicate that there is 'a need for biopsy and subsequent BRAF analysis of a metastatic lesion when treatment with kinase inhibitors is considered', even when biopsy of the primary lesion has previously been undertaken. (16)

Our own data suggest that the rate of true discordance of BRAF status between matched primary and metastatic melanoma tumours is low. Even among studies which have used IHC, a discordance rate of 0% is somewhat unusual, although it has occurred in two previous studies (see Table 2). (20, 21) However, our findings must be interpreted with caution in the context of earlier reports of the occurrence of melanoma metastases with BRAF status different to that of the primary tumour. On the other hand, our lab has significant experience in the use of the VE1 antibody for detection of V600E BRAF, and in our hands there is a high level of agreement between IHC for BRAF V600E detection and PCR methods. (13) This makes it unlikely that the absence of discordant cases in our series can be explained by technical issues with the IHC protocol.

Valachis and Ullenhag discuss the possibility that false negatives on PCR due to low tumour volume may explain the higher primary/metastatic BRAF discordance which is seen with PCR as compared to IHC. However, a secondary analysis of their data including only studies which reported an adequate number of tumour cells did not give significantly different results to their main analysis, which they regard as evidence that low tumour cell content in some samples does not contribute significantly to discordance between sites. (16) Nonetheless, our series includes a case in which there was apparent discordance on PCR (BRAF mutated primary tumour giving rise to BRAF wild type metastasis) which was not replicated by IHC and in which subsequent repeat PCR on the metastasis post-macrodissection was positive. Therefore it remains credible that



some instances of PCR discordance between sites could be due to technical issues related to laboratory methods employed. This complicates the interpretation of situations when two tumours from the same patient seem to have different BRAF status, and may be a major issue if testing of a metastatic site was to be required in all patients prior to initiation of targeted therapy, as has been proposed by some authors. (16) It should be noted that IHC with VE1 was positive at both sites in this case, demonstrating the usefulness of IHC as a complementary technique to PCR.

Our study has some limitations. Firstly, our sample size of 53 is relatively small, although only 4 larger IHC studies are documented in the literature (with 54, 64, 82 and 88 patients). It is possible that had more patients been included we would have found some instances of true discordance between primary and metastatic BRAF status. Secondly, we performed PCR on only one site in all cases except one, where discordance between PCR and IHC results was noted. Carrying out PCR on all cases would have allowed for a more complete molecular and immunohistochemical characterisation of our study group. However, as we have found IHC to be a reliable and robust method for detection of BRAF mutation, we consider this unlikely to be an issue. (13) Finally, the frequency of BRAF mutation positivity was low. 10 of 53 (19%) of cases showed evidence of BRAF V600E mutation on IHC. When one case which was positive for BRAF V600K mutation on PCR was considered the overall rate of activating BRAF mutation was 21%. While this is low compared to international estimates where approximately 40% of melanoma is typically BRAF mutated, previous work in the Irish population has suggested that the frequency of BRAF mutation in this group is lower, with a rate 24% reported in one of the largest studies. (37) However, it may be difficult to generalise our results to other populations with higher incidences of BRAF mutations in melanoma.

In conclusion, this study demonstrates that IHC for V600E BRAF expression using the VE1 antibody is a useful adjunct to PCR in assessing for the presence of the mutation melanoma and comparing its expression between primary lesion and distant metastases. While the finding of 100% concordance may not be replicated in future larger series or in other populations, taken in the context of previous studies our findings add further support to the conclusion that differences in BRAF status between primary and metastatic sites as determined by immunohistochemistry are significantly less common than discordance as assessed by PCR. (18-25) Our description of a case of apparent PCR discordance which was subsequently shown to be due to a false negative at one site suggests that some of this difference in findings between methods may be due to technical difficulties with obtaining accurate molecular data from small tumour deposits.

## Figure Legends

Figure 1: BRAF V600E immunohistochemistry staining: A) Weak 1+ positivity, 20x; B) Moderate 2+ positivity, 40x; C) Strong 3+ staining, 40x.



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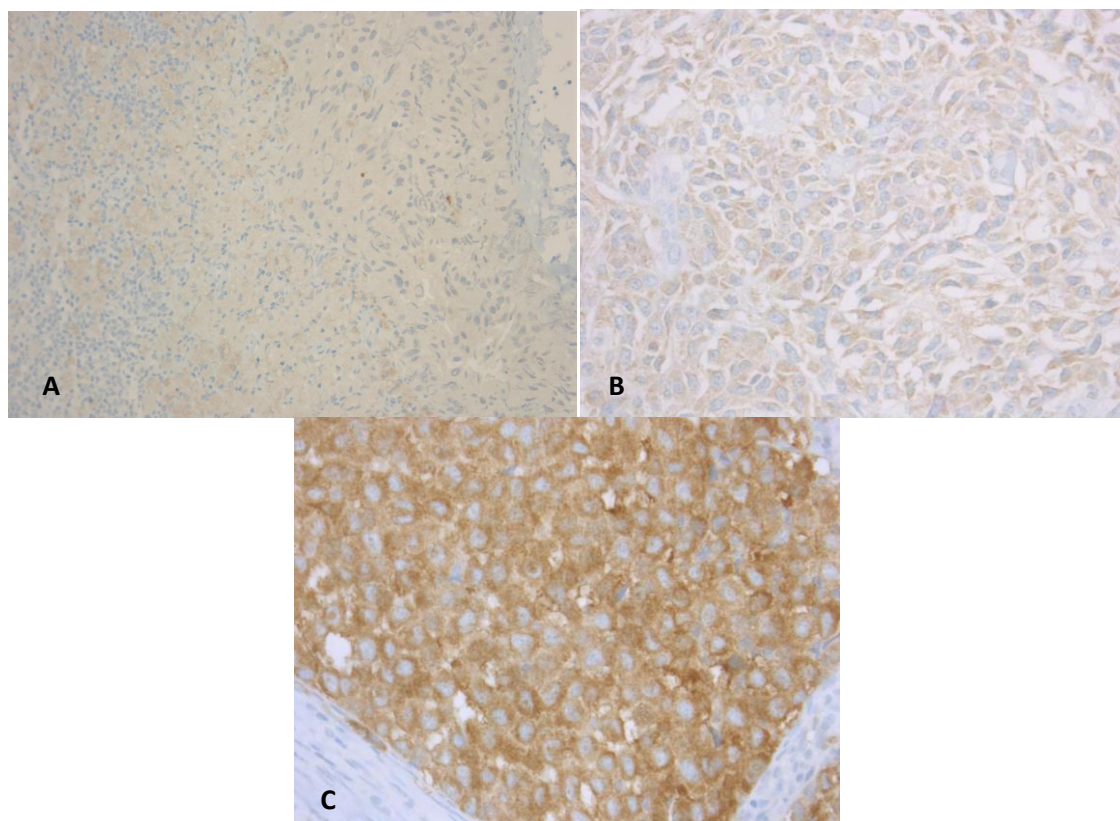


Figure 1: BRAF V600E immunohistochemistry staining: A) Weak 1+ positivity, 20x; B) Moderate 2+ positivity, 40x; C) Strong 3+ staining, 40x.

All Cases			BRAF Mutated Cases (by IHC of Primary)		BRAF Wild Type Cases (by IHC of Primary)	
<b>Median Patient Age</b>	69 years		37 years		70 years	
<b>Time Between Biopsies</b>	9 months (Range: 0 - 83 months)		8 months (Range: 0 - 27 months)		9 months (Range: 0 - 83 months)	
<b>Primary Site</b>	Limb	33 (62%)	Limb	4 (40%)	Limb	29 (67%)
	Head and Neck	10 (19%)	Head and Neck	3 (30%)	Head and Neck	7 (17%)
	Trunk	7 (13%)	Trunk	3 (30%)	Trunk	4 (9%)
	Mucosal surface	3 (6%)	Mucosal surface	0 (0%)	Mucosal surface	3 (7%)
	<i>Total</i>	<i>53 (100%)</i>	<i>Total</i>	<i>10 (100%)</i>	<i>Total</i>	<i>43 (100%)</i>
<b>Histological Subtype</b>	Nodular	17 (32%)	Nodular	2 (20%)	Nodular	15 (35%)
	Superficial Spreading	16 (30%)	Superficial Spreading	7 (70%)	Superficial Spreading	9 (21%)
	Acral Lentiginous	6 (11%)	Acral Lentiginous	0 (0%)	Acral Lentiginous	6 (14%)
	Lentigo Maligna Type	5 (9%)	Lentigo Maligna Type	0 (0%)	Lentigo Maligna Type	5 (12%)
	Not specified	9 (17%)	Not specified	1 (10%)	Not specified	8 (19%)
	<i>Total</i>	<i>53 (100%)</i>	<i>Total</i>	<i>10 (100%)</i>	<i>Total</i>	<i>43 (100%)</i>
<b>Metastatic Site</b>	Lymph node	33 (62%)	Lymph node	8 (80%)	Lymph node	25 (58%)
	Subcutaneous tissue	10 (19%)	Subcutaneous tissue	2 (20%)	Subcutaneous tissue	8 (19%)
	Skin	6 (11%)	Skin	0 (0%)	Skin	6 (14%)
	Salivary gland	2 (4%)	Salivary gland	0 (0%)	Salivary gland	2 (5%)
	Lung	1 (2%)	Lung	0 (0%)	Lung	1 (2%)
<i>Total</i>			<i>Total</i>	<i>10 (100%)</i>	<i>Total</i>	<i>43 (100%)</i>

		(100%)				
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Table 1: Pathological characteristics of the study cohort

*Table 2:* A summary of all previous studies investigating the rate of discordance between melanoma primary and metastases by either polymerase chain reaction (PCR) or immunohistochemistry (IHC).

Author	Method of BRAF Assessment (IHC/PCR/PCR & IHC)	Cohort Size	Rate of Discordance by PCR	Rate of Discordance by IHC
Bourault <i>et al</i> , 2014 <sup>18</sup>	PCR & IHC	88	4.5%	4.5%
Bradish <i>et al</i> , 2015 <sup>28</sup>	PCR	25	16%	-
Colombino <i>et al</i> , 2013 <sup>27</sup>	PCR	236	11.8%	-
Eriksson <i>et al</i> , 2015 <sup>19</sup>	IHC	63	-	3%
Heinzerling <i>et al</i> , 2013 <sup>29</sup>	PCR	53	18.9%	-
Houben <i>et al</i> , 2004 <sup>30</sup>	PCR	24	17%	-
Kaji <i>et al</i> , 2017 <sup>25</sup>	PCR	17	47%	-
Manfredi <i>et al</i> , 2016 <sup>20</sup>	PCR & IHC	35	6%	0%
Menzies <i>et al</i> , 2014 <sup>21</sup>	IHC	64	-	0%
Nardin <i>et al</i> , 2015 <sup>31</sup>	PCR	25	8%	-
Omholt <i>et al</i> , 2003 <sup>32</sup>	PCR	51	4%	-
Riveiro-Falkenbach <i>et al</i> , 2015 <sup>33</sup>	PCR	140	16%	-
Saint-Jean <i>et al</i> , 2014 <sup>34</sup>	PCR	30	7%	-
Shinozaki <i>et al</i> , 2004 <sup>35</sup>	PCR	13	38%	-
Saroufim <i>et al</i> , 2014 <sup>22</sup>	IHC	40	-	27.5%
Verlinden <i>et al</i> , 2014 <sup>23</sup>	IHC	30	-	7%
Yancovitz <i>et al</i> , 2012 <sup>26</sup>	PCR	18	44%	-
Yaman <i>et al</i> , 2016 <sup>24</sup>	IHC	48	-	14.7%